EXHIBIT B

JOURNAL OF VIROLOGY, Aug. 2002, p. 7736–7745
0022-538X/02/$04.00+0 DOI: 10.1128/JVI.76.15.7736–7746.2002 Vol. 76, No. 15

Cell Clones Selected from the Huh7 Human Hepatoma Cell Line Support Efficient Replication of a Subgenomic GB Virus B Replicon

Amedeo De Tomassi, Maura Pizzuti, Rita Graziani, Andrea Sbardellati, Sergio Altamura, Giacomo Phonessa, and Cinzia Traboni*

Istituto di Ricerche di Biologia Molecolare P. Angeletti (IRBM), 00040 Pomezia (Rome), Italy

Received 30 January 2002/Accepted 30 April 2002

Tamarins (*Saguinus* species) infected by GB virus B (GBV-B) have recently been proposed as an acceptable surrogate model for hepatitis C virus (HCV) infection. The availability of infectious genomic molecular clones of both viruses will permit chimeric constructs to be tested for viability in animals. Studies in cells with parental and chimeric constructs would also be very useful for both basic research and drug discovery. For this purpose, a convenient host cell type supporting replication of in vitro-transcribed GBV-B RNA should be identified. We constructed a GBV-B subgenomic selectable replicon based on the sequence of a genomic molecular clone proved to sustain infection in tamarins. The corresponding in vitro-transcribed RNA was used to transfect the Huh7 human hepatoma cell line, and intracellular replication of transfected RNA was shown to occur, even though in a small percentage of transfected cells, giving rise to antibiotic-resistant clones. Sequence analysis of GBV-B RNA from some of those clones showed no adaptive mutations with respect to the input sequence, whereas the host cells sustained higher GBV-B RNA replication than the original Huh7 cells. The enhancement of replication depending on host cell was shown to be a feature common to the majority of clones selected. The replication of GBV-B subgenomic RNA was susceptible to inhibition by known inhibitors of HCV to a level similar to that of HCV subgenomic RNA.

A problem that remains unsolved in the search for new therapeutic agents against hepatitis C virus (HCV) infection (12, 20, 25) is the availability of a small-animal model suitable for pharmacological studies, since the known host range of this virus includes only humans and chimpanzees. An interesting murine model based on the repopulation of mouse liver with human HCV hepatocytes has recently been proposed as sustaining HCV infection and yielding significantly high serum titers (17). The current version of this model, however, presents several drawbacks, including the difficulty of identifying an unlimited source of human hepatocytes and the low success rate, due in part to the peculiar genetic background of the mice used. Albeit very promising, this model needs improvement to be turned into a method accessible to most of the laboratories concerned.

In the absence of a direct and convenient small-animal model, over the last few years a model for HCV infection alternative to the chimp has been proposed by various research groups. That model is based on the use of a surrogate virus-host system, tamarins (*Saguinus* species) infected by GB virus B (GBV-B). A growing body of data about the enzymatic activity of GBV-B proteins has corroborated the hypothesis that useful information for research on anti-HCV drugs can be derived from experiments with GBV-B in tamarins. The identification of an infectious cDNA has provided an indispensable tool to engineer the virus genome by insertion of HCV regions of interest. The availability of a small nonhuman primate GBV-B host and the perspective of using chimeric HCV/GBV-B viruses open up possibilities for pharmacology studies on novel anti-HCV therapies that perform better than the current treatment.

The similarity between the genome organizations of HCV and GBV-B was underlined upon discovery of GBV-B in 1995 (19, 31). The first experimental demonstration of this similarity at the functional level involved the enzymatic activity of NS3 protease (29). More detailed analyses of the polyprotein processing followed, outlining the functional relationship between the two viruses' NS3 proteins, which are active on reciprocal substrates but require their own cognate NS4A cofactors (6, 26). The helicase and NTPase activities associated with the C-terminal domain of GBV-B NS3 protein were also reported as comparable to those of HCV (37). The RNA-dependent RNA polymerase (RdRp) activity encoded by a truncated form of GBV-B NS5B gene was analyzed in in vitro studies (38; L. Tomei, unpublished data), showing that the similarity of this key enzyme in the two viruses also encourages the use of this surrogate model. Finally, evidence has been published confirming that the GBV-B and HCV untranslated regions (UTRs), which play an important role in initiation of the replication process via interactions with viral proteins such as helicase and RdRp, have common features. The internal ribosome entry site (IRES)-containing 5′ UTR of GBV-B shows striking structural and functional similarity to that of HCV (8, 23, 24). The 3′ end of the 3′ UTR has a secondary structure similar to that of HCV (27) and, as in the case of HCV, is indispensable for replication and in vivo infectivity (5, 28).

GBV-B infection of tamarins has been achieved by intrahepatic injection of RNA in vitro transcribed from genomic molecular constructs (5, 28), providing information about the in-

* Corresponding author. Mailing address: Istituto di Ricerche di Biologia Molecolare P. Angeletti (IRBM), Via Pontina Km 30,600, 00040 Pomezia (Rome), Italy. Phone: 39 06 91093241. Fax: 39 06 91093654. E-mail: cinzia_traboni@merck.com.

fectious GBV-B sequences suitable as scaffolds to produce chimeric genomes bearing HCV genes. The possibility of infecting tamarins with chimeric viruses represents the optimal approach to the GBV-B model, since the true HCV target of interest would be included in the surrogate infectious agent. However, although producing such chimeras now seems feasible, presumably a number of constructs will have to be tested before viable molecules can be identified.

In view of this, the availability of a cell-based system to check the infectivity and/or replication of molecular constructs based on the GBV-B genome would be important to run preliminary experiments with molecular constructs before testing them in animals. This would satisfy both ethical and practical requirements, as a number of animals would be spared because constructs unable to sustain replication would be discarded by pretesting in cells. Also, a number of preliminary pharmacology experiments could be run directly in cells bearing replication-competent chimeric RNA. For these reasons, the recently published infection system for GBV-B based on tamarin primary hepatocytes (2) and its use to test the effect of antiviral agents (14) are of great interest but still present limitations intrinsic to the use of primary cells. Even though this system helps to reduce the number of tamarins utilized for in vivo experiments, it still implies euthanizing tamarins to produce the cells. Moreover, primary hepatocytes are not easy to prepare and handle and might also present problems in experimental reproducibility. Another potential limitation of the tamarin hepatocyte infection system might be the difficulty of transfecting RNA from recombinant constructs, which would be important for a cell-based analysis of viability of chimeric molecules. No data are available at the moment about attempts in this direction.

With the aim of establishing a system for GBV-B replication in cell lines amenable to genetic studies, we have constructed a selectable GBV-B subgenomic replicon and shown that it replicates in human hepatoma cell line Huh7. The observed replication does not require adaptive mutations with respect to a genomic construct that replicates in tamarins and only occurs in a fraction of the Huh7 cells able to enhance the replication level above the detection threshold.

MATERIALS AND METHODS

Cell lines and culture conditions. Human hepatoma cell lines Huh7, HepG2, and HepBR (our laboratory stocks) were grown in high-glucose Dulbecco's modified Eagle's medium (Life Technologies) supplemented with 2 mM L-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 10% fetal bovine serum. Cells were subcultivated twice a week with a 1:5 split ratio. *Aotus nitrigulus* (owl monkey) kidney cell line OMK 637-60 (ATCC CRL-1556) was grown in minimal essential medium in Earle's balanced salt solution with nonessential amino acids (MEM; Life Technologies) supplemented with 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 10% fetal bovine serum. *Saguinus oedipus* (tamarin) lymphoblast cell line B93-8a, kindly provided by F. Kohune, was grown in RPMI 1640 medium (RPMI; Life Technologies) supplemented with 2 mM L-glutamine, 100 U of penicillin/mL, 100 μg of streptomycin/ml, 10 mM HEPES, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. HCV replicon-bearing cell line HBI10A was kindly supplied by C. Migliaccio. Neomycin-resistant lines were grown in the presence of G418 at concentrations ranging between 0.250 and 1 mg/ml.

Plasmid construction. The neo-RepB GBV-B replicon construct was obtained by replacing the regions coding for the structural proteins (starting from nucleotide 25 of the core protein gene) and N52 protein with the sequences of the neomycin phosphotransferase (*neo*) gene and encephalomyocarditis virus (EMCV) IRES in plasmid FL-J/pACYC177 (accession number AJ277947), encoding a GBV-B infectious full-length cDNA downstream of the T7 polymerase promoter. The GBV-B neomycin phosphotransferase gene and EMCV IRES were amplified by PCR from the pHCVNeo17.wt plasmid (18), kindly supplied by C. Migliaccio, and joined cropwise to the GBV-B sequences by assembly PCR and ligation reactions, creating a unique *AscI* site upstream of the neomycin phosphotransferase gene. The neo-RepB final replicon sequence was moved as a *BamHI-XhoI* fragment into the more versatile pGBT9 vector (Clontech). Two *SapI* sites were then removed from the neomycin phosphotransferase gene by primer-based mutagenesis, leaving a *SapI* site at the 3′ end of the GBV-B coding sequence to be used for runoff transcription.

The bla-RepB replicon was constructed by replacing the *AscI-PmeI* fragment spanning the neomycin phosphotransferase gene in the GBV-B neo-RepB construct with an *AscI-PmeI* fragment including the β-lactamase (*bla*) gene from a plasmid kindly provided by J. Grobler. Mutations in the polymerase active-site GDD motif were obtained by first constructing a GDD-to-GAA-mutated neo-RepB clone by means of primer-based mutagenesis and subsequently replacing a restriction fragment spanning the mutation onto plasmid bla-RepB. Reverse transcription (RT)-PCR amplification products of RNA from HAB.1/Huh7 cells were cloned into the pCR2.1 vector to perform sequencing.

Sequence analysis. Sequencing was performed by the Big Dye terminator cycle sequencing kit with AmpliTaq (Applied Biosystems) and run with an Applied Biosystems model 373A sequencer.

In vitro transcription. *SapI*-linearized plasmids encoding GBV-B replicons were in vitro transcribed by T7 RNA polymerase with an Ambion Megascript kit under nuclease-free conditions following the manufacturer's instructions. The reaction was terminated by incubation with DNase I and precipitation with LiCl according to the manufacturer's instructions. RNA was resuspended in nuclease-free water, quantified by absorbance at 260 nm, immediately frozen in dry ice in 10-μg aliquots, and stored at −80°C.

Transfection of GBV-B replicon RNA and monitoring of replication. Human hepatoma HepBR, HepG2, Huh7, and Huh7-derived cell lines as well as monkey cell lines were used to test replication of GBV-B replicon constructs. Confluent cells from 15-cm-diameter plates were split 1:2. Cells were recovered after 24 h in 5 ml of medium, washed twice with 40 ml of cold diethylpyrocarbonate-treated phosphate-buffered saline (PBS), filtered with Cell Strainer filters (Falcon), and diluted in cold diethylpyrocarbonate-treated PBS at a concentration of 10^7 cells/ml. Then 2×10^6 cell aliquots were subjected to electroporation with 10 μg of in vitro-transcribed RNA by two pulses at 0.35 kV and 10 μF with a Bio-Rad Gene pulser II. Immediately after electric pulses, cells were diluted in 8 ml of complete Dulbecco's modified Eagle's medium and processed with different protocols depending on the selection and tracer used.

In the case of transformation with constructs bearing the neomycin phosphotransferase gene, cells were divided among three plates of 15 cm in diameter, and on the following day, the selecting antibiotic G418 (Sigma G-9510) was added at concentrations ranging between 0.250 and 1 mg/ml. G418-sensitive cells died in 2 weeks, and at the fourth week, surviving cell clones could be picked from the cultures with 0.250 mg of G418/ml and expanded by growth in individual plates. When the *bla* reporter gene was used, 1.5, 1.0, and 0.5 ml of transfected cell suspension was plated in each well of six-well plates (Falcon) to be stained at 24, 48, and 72 h, respectively, with the CCF2 substrate system (Aurora Biosciences Corporation). When quantitative PCR was used to measure transient replication after transfection of either bla-RepB or neo-repB RNA, 1×10^5 to 2×10^5 cells/well were plated in six-well plates. After 3 days, total RNA was purified as described in the Trizol protocol (Life Technologies), and 10 μl out of 100 μl of total RNA recovered was used in each TaqMan reaction.

Preparation of proteins, genomic DNA, and total RNA. Total RNA, genomic DNA, and total proteins were purified from cells grown in monolayer with the Trizol reagent (Life Technologies), following the manufacturer's instructions.

Northern blot. For Northern blots, 10 μg of total RNA extracted from Huh7-derived cells was subjected to electrophoresis on a 1% agarose-formaldehyde gel, blotted onto Amersham Hybond-N$^+$ membranes, and hybridized to a GBV-B RNA probe. Electrophoresis, blotting, and hybridization procedures were performed by following the Hybond-N$^+$ membrane instruction manual with slight modifications. The [α-^{32}P]CTP-labeled RNA probe was produced by in vitro transcription of a GBV-B genomic fragment (nucleotides 4641 to 6060 of the genomic sequence) cloned in the pCR2.1 vector under the T7 promoter in the orientation producing a negative-stranded transcript.

Nonquantitative RT-PCR. Total RNA was used for first-strand cDNA synthesis by Superscript II reverse transcriptase (Gibco-BRL) under the manufacturer's conditions. PCR amplification was performed with Elongase enzyme mix (Gibco-BRL) or *Taq* DNA polymerase (Promega). Primers were purchased from MWG (Ebersberg, Germany).

TaqMan quantification of GBV-B RNA. GBV-B RNA was quantified by a real-time, 5′-exonuclease PCR (TaqMan) assay with a primer-probe set that recognized a portion of the GBV-B 5′ untranslated region (5′ UTR). The primers GBV-B-F3 (GTAGGGXXXXGGGACTCAT) and GBV-B-R3 (TCAGG GCCATCCAAGTCAA) and probe GBV-B-P3 (6-carboxyfluorescein-TCGCG TGATGACAAGCGCCAAG-*N,N,N′,N′*-tetramethyl-6-carboxyrhodamine) were selected with the Primer Express software (PE Applied Biosystems). The primers were used at 10 pmol/50-μl reaction volume, and the probe was used at 5 pmol/50-μl reaction volume. The reactions were performed with a TaqMan Gold RT-PCR kit (PE Applied Biosystems) and included a 30-min reverse transcription step at 48°C, followed by 10 min at 95°C and by 40 cycles of amplification with the universal TaqMan standardized conditions (15 s of denaturation at 95°C followed by a 1-min annealing/extension step at 60°C).

RNA transcribed from a plasmid containing the first 2,000 nucleotides of the GBV-B genome was used as a standard to establish genome equivalents. Standard RNA was transcribed with a T7 Megascript kit (Ambion) and was purified by DNase treatment, phenol-chloroform extraction, Sephadex G-50 filtration, and ethanol precipitation. RNA was quantified by absorbance at 260 nm and stored at −80°C. All reactions were run in duplicate with the ABI PRISM 7700 sequence detection system (PE Applied Biosystems). The PE Applied Biosystems specific primer set was used to quantify the endogenous reference human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Whenever necessary, RNA extracted from cells transfected with RNA of GAA mutant constructs was used as the calibrator. Results from two independent experiments were analyzed with both the comparative threshold cycle (Ct) method and the standard curve method.

Test of putative inhibitors of GBV-B replication. Huh7 cell clones carrying GBV-B or HCV replicons were used to test the effect of putative inhibitors on replication. A total of 10^4 cells were plated into each well of a series of wells of six-well plates (Falcon) in medium without G418. After 16 h, the medium was discarded, and increasing concentrations of the test compound in fresh medium were added to each series of wells. Controls were run with the specific compound solvent at the same concentration used to test individual compounds (1× culture medium for alpha interferon [IFN-α] and 0.25% dimethyl sulfoxide in the other cases). Cells were grown for up to 3 days in the presence of compounds or solvents, avoiding cell confluence, and finally lysed with Trizol. Total RNA was purified as described in the Trizol protocol (Life Technologies); 10 μl out of 100 μl of total RNA was used in each reaction.

TaqMan analysis was performed with the neomycin primer set (aq NEO 1 (OATGGATTGCACGCAGGTTC) and taq NEO 5 (CCCACTCATAGCCGAA TAGCC) and probe NEO probe 1 (6-carboxyfluorescein-TCCGCCCCTTCG GTGGAG-*N,N,N′,N′*-tetramethyl-6-carboxyrhodamine). Human GAPDH mRNA, quantified with a specific primer set (PE Applied Biosystems), was used as an endogenous reference; GBV-B RNA extracted from mock-treated cells was used as the calibrator. Results from two independent experiments were analyzed with both the comparative Ct method and the standard curve method.

Toxicity was also routinely checked by the colorimetric cell assay. This assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product, which is insoluble in water. The amount of formazan produced is directly proportional to the number of living cells. Human IFN-α2b (Intron A3) and ribavirin (1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide) were purchased from Schering-Plough. mycophenolic acid was obtained from Sigma, and 4-{[(2*E*)-3-phenylprop-2-enyl]oxy}benzaldehyde thiosemicarbazone was kindly provided by S. Harper.

Western blot. Protein extracts were prepared from 10^6 cells by Trizol extraction and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide) (3 h at 100 μg/slot). Purified NS3 and NS5B prepared as described before (28) were used as controls. The gel was blotted onto a nitrocellulose filter by routine methods. The filter was incubated with a 1:50 dilution of a pool of four tamarin sera previously shown to be immunoreactive against GBV-B antigens (28) in blocking buffer (5% nonfat dry milk, 0.05% Tween 20 in Tris-buffered saline). The filter was washed five times with blocking buffer and incubated with a mouse anti-monkey immunoglobulin antibody (Sigma). After five more washes, the filter was incubated with a horseradish peroxidase-conjugated mouse antibody and treated with West Pico Supersignal chemiluminescent substrate (Pierce) following the manufacturer's instructions, and the signals were detected by X-ray film exposure.

Nucleotide sequence accession number. The nucleotide sequence of the GBV-B neo-RepB construct reported in this paper has been deposited in the DDBJ/EMBL/GenBank database under accession number AJ428935.

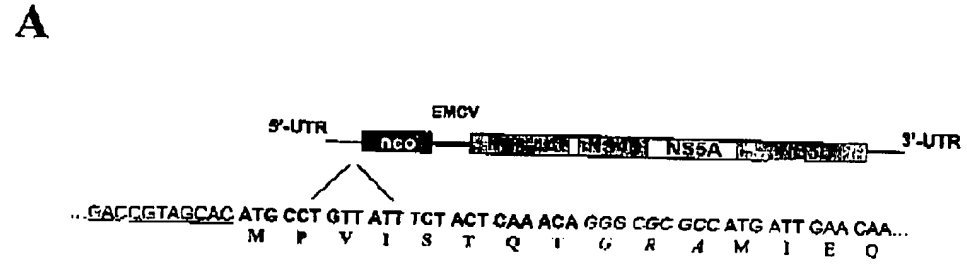
RESULTS

Cloning GBV-B neomycin-resistant subgenomic replicons. Plasmid FL-3, encoding the GBV-B genomic sequence and previously proven infectious in tamarins (28), was used as a parental molecule to build the GBV-B subgenomic replicon constructs. The GBV-B bicistronic neo-RepB replicon was designed as schematized in Fig. 1A, with the same gene arrangement described for HCV replicons (4, 16). In the first cistron, the GBV-B 5′ UTR sequence directs translation of the neomycin phosphotransferase selectable marker; the second cistron is formed by the EMCV IRES directing translation of the GBV-B nonstructural proteins from NS3 to NS5B. Downstream of the coding region is the complete GBV-B 3′ UTR sequence (*ZI*). A stretch of 24 nucleotides of core coding sequence was included in the construct downstream of the GBV-B 5′ UTR. The N terminus of the neomycin phosphotransferase protein resulting from the described cloning design is fused to the first eight amino acids of GBV-B core protein and to three more amino acids generated by the addition of a cloning site upstream of the neomycin phosphotransferase gene.

The neo-RepB plasmid was in vitro transcribed after linearization at an engineered *Sap*I site to generate GBV-B subgenomic transcripts terminating at the precise 3′ end corresponding to that of the genomic infectious molecules. In vitro-transcribed RNA was transfected into Huh7 human hepatoma cells by electroporation. RNA from the neo-RepB-GAA plasmid, mutated in the active site of the NS5B polymerase, was used as a negative control. After 24 h, selection was applied to the transfected cells by growing them in the presence of 0.250 mg of G418/ml. The majority of the cells did not develop resistance to the selecting agent, but in the long run it was possible to identify neomycin-resistant cell clones, which were picked after 30 days of culture and grown as individual cell lines. In typical experiments, we selected from 100 to 800 neomycin-resistant colonies upon transfection of 2×10^6 cells with 10 μg of neo-RepB RNA, depending on the efficiency of the individual transfection experiment. Parallel attempts at isolating neomycin-resistant clones upon selection with higher G418 concentrations failed, whereas it was possible to increase the concentration of G418, for at least some of the cell clones, once the clones had been isolated and individually grown as cell lines. The individual cell lines showed some variability in growth rate; for most of the subsequent experiments we used B76.1/Huh7, a cell line derived from one of the fast-growing original clones called B76/Huh7 and kept in 1 mg of G418 per ml. Transfection of RNA from a replicon construct lacking the region encoding the N terminus of core protein gave a 12-fold-lower number of colonies showing slow growth, which were not isolated and individually characterized.

Attempts to reproduce successful transfection of GBV-B subgenomic replication with other human hepatoma cell lines such as HepG2 and Hep3B as recipients failed (data not shown). Similar experiments performed with primate (tamarin and owl monkey) cell lines of nonhepatic origin were also unsuccessful (data not shown).

Detection and quantification of GBV-B RNA in Huh7 cells transfected with the GBV-B replicon. To demonstrate the presence of GBV-B RNA in transfected G418-resistant cells, total



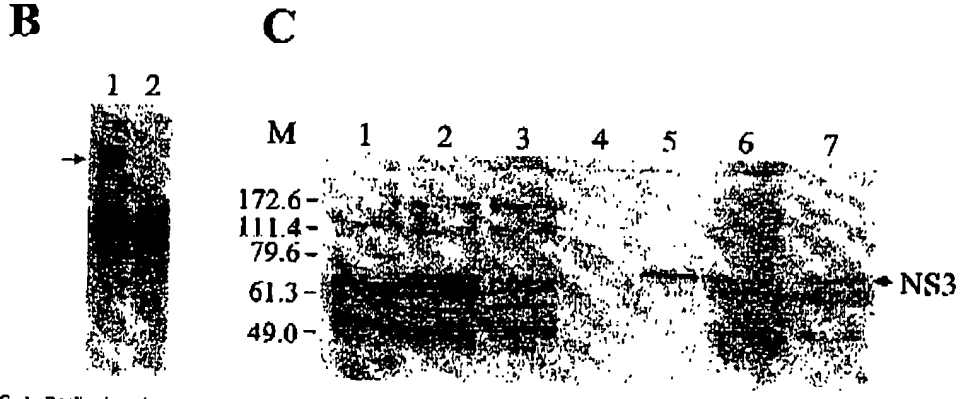


FIG. 1. Replication of subgenomic GBV-B replicon. (A) Schematic representation of GBV-B neo-RepB construct. The nucleotide sequence below the drawing corresponds to the sequence of the boundary between the GBV-B 5′ UTR and the neomycin phosphotransferase gene. The portion of the sequence belonging to the GBV-B 5′ UTR is underlined, and that representing translated GBV-B sequences is boldfaced. The sequence corresponding to a *Pme*I restriction site is in italics, and that corresponding to the neomycin phosphotransferase gene is in roman type. The translated sequences are organized in nucleotide triplets, and the corresponding amino acids are indicated below each cognate nucleotide sequence. (B) Detection of replicon RNA by Northern blotting. Total RNA extracted from replicon-bearing cell line B76.1/Huh7 was subjected to Northern blot analysis with a ^{32}P-labeled probe spanning part of the nonstructural region of GBV-B. Autoradiography was obtained by 4 days of exposure with Hyperfilm MR Kodak films. Lane 1, RNA from cell line B76.1/Huh7; lane 2, RNA from negative control cell line eB76.1/Huh7. The arrow indicates the position of the neo-RepB RNA marker. (C) Detection of GBV-B proteins in Huh7 cell clones supporting GBV-B replication. Protein extracts of replicon clones were subjected to Western blotting with serum of tamarins infected with GBV-B. Lane 1, extract from GBV-B replicon cell line B78/Huh7; lane 2, extract from cell line B76/Huh7; lane 3, extract from untransfected Huh7 cells; lane 4, purified GBV-B NS5B protein; lane 5, purified GBV-B NS3 protein; lane 6, extract from cell line B86/Huh7; lane 7, extract from cell line B39/Huh7. The position of the NS3 protein is indicated.

RNA was extracted from the B76.1/Huh7 cell line and control cells and analyzed by Northern blotting (Fig. 1B). A specific hybridization signal at the expected molecular weight was detected only in B76.1/Huh7 (Fig. 1B, lane 1) with an RNA probe corresponding to a portion of the GBV-B genome.

The presence of replicon RNA molecules in the neomycin-resistant cell lines was also confirmed by PCR. Nonquantitative RT-PCR was performed with various sets of primers (data not shown); PCR products were obtained only when a reverse transcription step was included, indicating that amplification was exclusively RNA dependent and not due to the presence of residual DNA in the RNA preparation. Moreover, the integration of replicon copies in host genomic DNA was also excluded by lack of amplification of both GBV-B and *neo* gene sequences from genomic DNA preparations (data not shown).

To obtain quantitative measurements of replicon RNA molecules in the neomycin-resistant clones, TaqMan RT-PCR with primers and probes complementary to the GBV-B 5′ UTR region was performed with accurate experimental procedures and calculations as described in Materials and Methods. The results, summarized in Table 1, confirmed the RNA-dependent amplification of replicon sequences and showed that the number of GBV-B genome equivalents/cell was variable, ranging between 30 and 100, a number considerably lower than that obtained with HCV replicons (4, 16). The higher G418 concentration resulted in a threefold increase in

TABLE 1. Comparison of neo-RepB copy numbers in individual neomycin-resistant cell lines[a]

Cell line	Genome equivalents/μg of cell RNA	Mean genome equivalents/cell
B4	3.24×10^6	32.4
B57	3.42×10^6	103.0
B59	2.68×10^6	80.5
B76	1.40×10^6	35.0
B76.1*	4.85×10^6	121.0
B78	1.30×10^6	38.9
B86	0.58×10^6	29.3

[a] Cell lines were grown at 0.250 or 1 (*) mg of G418 per ml. The amount of total cellular RNA was measured by determining absorbance at 260 nm.

the replicon RNA amount, as shown in Table 1 for clones B76/Huh7 and B76.1/Huh7.

Detection of GBV-B proteins. To directly visualize GBV-B proteins produced from replicon clones, we performed Western blotting analyses with extracts of individual cell clones. A pool of four GBV-B-infected tamarin sera in which seroconversion had already been detected (28) was used as a source of antibodies. The results showed a specific band at the expected molecular weight for NS3 (Fig. 1C, lanes 1, 2, 6, and 7) that was not present in the mock-transfected cells (Fig. 1C, lane 3). Its identity as NS3 protein was confirmed by the use of a purified NS3 preparation as a positive control (Fig. 1C, lane 5). Reactivity of the tamarin serum pool to NS5B, which is similar in size to NS3, was excluded by the lack of signal with purified NS5B used as a positive control (Fig. 1C, lane 4). This was in agreement with previous data obtained with these sera, showing antibodies to NS5B protein by enzyme-linked immunosorbent assay (ELISA) but not by Western blotting (28). The data were confirmed with rabbit polyclonal sera against NS3 and NS5B, but with a higher background (data not shown).

GBV-B replicon RNA molecules that replicate in Huh7 cells show no sequence variation from the parental full-length infectious RNA. The yield of neomycin-resistant clones obtained upon transfection of GBV-B replicon RNA in Huh7 cells was significantly lower (1 to 8 clones/10,000 cells used for transfection) than expected from electroporation efficiency. This led us to think that for GBV-B, as observed for the HCV replicon (4, 15; G. Paonessa, unpublished data), successful transfection leading to the formation of cell clones supporting replication was determined by rare events affecting either the transfected RNA or the recipient cells.

To test whether adaptive mutations in the replicon sequence might be responsible for increased replication efficiency, portions of the GBV-B replicon spanning the complete sequence were amplified by RT-PCR of total RNA extracted from the B76.1/Huh7 cell line and subcloned for sequencing. Two subclones per region, obtained from independent RT-PCRs, were sequenced. Besides sporadic mutations present only in one of the two subclones of each region (probably due to PCR errors), no mutation was consistently found in both individual subclones analysed per region. We cannot exclude that a mixed replicon RNA population in which no mutation is present in every molecule exists in this cell line. However, the most probable explanation is that the sequence of the replicating GBV-B replicon molecules is indeed identical to that of the RNA transcribed from the neo-RepB construct and originally transfected in recipient Huh7 cells. This interpretation was confirmed by direct sequencing of PCR fragments spanning the whole sequence of GBV-B amplified from RNA extracted from two more cell clones, demonstrating that the absence of adaptive mutations is a general feature of this system.

We matched the positions of adaptive mutations reported for the HCV replicon (1) with corresponding amino acid residues in the sequence deduced for the corresponding GBV-B proteins. The results reported in Fig. 2 show the presence in GBV-B of "HCV-adapted" amino acids in only one case, mutation R2884G mapped in HCV NS5B (15), since a glycine residue is located in the corresponding GBV-B position. It has to be said that the sequence homology between HCV and GBV-B proteins, while significant and validated by functional assays for NS3 and NS5B, is not satisfactory for NS5A, where the majority of adaptive mutations in the HCV replicon have been mapped.

Efficient transduction and replication of GBV-B replicons depends on permissiveness of host cells. Sequencing of the GBV-B replicon from neomycin-resistant cell lines had ruled out the hypothesis that adaptive mutations were necessary to obtain replication. We then experimentally tested the hypothesis that the low percentage of Huh7 cells sustaining GBV-B replication was due to selection of permissive host cells present in the originally transfected Huh7 population. We decided to remove the replicon RNA from the B76.1/Huh7 clone and compare the ability of "cured" cells and parental Huh7 cells to support GBV-B replication upon ex novo transfection of replicon RNA.

In order to obtain a recipient cell line containing no residual replicon RNA molecule, we treated B76.1/Huh7 cells with human IFN-α at a high concentration and for a time sufficient to achieve total inhibition of replication joined to complete degradation of the resident GBV-B replicon RNA molecules. To this aim, the cell line B76.1/Huh7 was maintained in culture with 100 U of IFN-α per ml for 15 days without G418. The absence of the selectable RNA replicon molecules was checked at the end of the treatment by TaqMan analysis (data not shown) and by Northern blotting (Fig. 1B, lane 7) and confirmed by the inability of the IFN-treated clone to grow in the presence of the selector G418 at a range of concentrations from 0.250 to 1 mg/ml (data not shown).

The resulting cured cell line, cB76.1/Huh7, was then transfected with RNA in vitro transcribed from the neo-RepB plasmid and kept under G418 selection. In this case most of the cells used for transfection reacquired resistance to neomycin and survived, probably all those in which RNA uptake had occurred. To monitor the increased efficiency in transient replication of RepB RNA in cB76.1/Huh7 cells compared to wild-type Huh7 cells, we used a colorimetric assay (O. Flores, unpublished data) based on the development of a blue tracer depending on β-lactamase activity encoded by the transfected RNA transcribed from the bla-RepB plasmid, a version of RepB which expresses the marker β-lactamase in place of the selector neomycin phosphotransferase. The β-lactamase reporter system is very convenient because it allows direct visualization of individual positive cells with a quick assay and is also suitable to derive information about the amount of reporter present in individual cells by observing stain intensity.

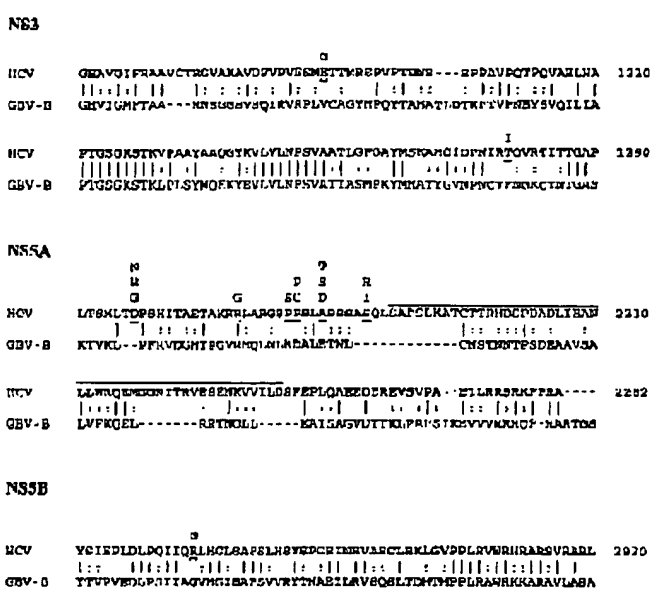


FIG. 2. Alignment of deduced amino acid sequences of HCV and GBV-B replicons spanning HCV adaptive mutations. Portions of the NS3, NS5A, and NS5B protein sequences are shown, derived from a unique alignment of the complete NS3-NS5A polyprotein regions. The position of the HCV mutation reported in the literature (1) is underlined in the wild-type sequence, and the mutated amino acids described in those positions are indicated above the wild-type sequence. The line above the HCV NS5A sequence indicates the amino acids missing in a deletion mutant (4). Numbering of HCV amino acids is as described in reference 1. The R2884G mutation in NS5B is boldfaced. Sequence homology analysis was performed with the PileUp program (Wisconsin package, version 9.1; Genetics Computer Group, Madison, Wis.).

The results obtained with the β-lactamase system at short times posttransfection and in the absence of selection substantially confirmed the trend observed after much longer times with G418-based selection. The number of blue-stained cells observed by transfecting cured cells was significantly higher than in the parental cell line, and the intensity and brightness of the color were also enhanced (Fig. 3A). Upon IFN-α treatment of bla-RepB-transfected cB76.1/Huh7 cells, no blue-stained cells were detectable, demonstrating that the stain was actually dependent on RepB replication (see Fig. 3A).

Quantitative analysis of transient replication was also performed by real-time TaqMan 3 days after transfection, showing a 20- to 30-fold increase in RepB RNA compared to a nonreplicating control in cB76.1/Huh7 cells, whereas RepB RNA was below the detection limit in uncured Huh7 cells. These data suggest that the individual cell giving rise to clone B76.1/Huh7 was capable of sustaining replication differently from the majority of the other cells in the originally transfected Huh7 population. The amount of specific RNA detected upon transfection of RNA from a variant construct lacking part of the core-coding region, though variable, was lower than that obtained with the original RepB construct (data not shown).

The "curing" procedure, though producing accurate results and yielding the cB76.1/Huh7 cured cell line itself, required a considerable amount of work and time, especially when coupled with the TaqMan assay as a read-out of the experiment. This led us to devise different strategies to track the frequency of the GBV-B replication enhancement due to host cell factors among the isolated neomycin-resistant clones. We decided to use bla-RepB RNA expressing β-lactamase as a reporter, detectable with a fast assay, to retransfect neomycin-resistant individual cell lines. To this aim, we kept eight independent neo-RepB cell lines for 2 weeks in the absence of G418 and transfected them with RNA transcribed from the bla-RepB plasmid. All cell lines supported replication with an efficiency considerably higher than that shown by the original nonclonal Huh7 population (0.005% blue cells, 3% pale blue cells), ranging from 10 to 80% blue cells, and with a certain degree of variability in the stain intensity among the different clones (see examples in Fig. 3B). This indicates that most of the original neomycin-resistant clones actually originated from the selection of transfected cells present in the total Huh7 population that were able to augment replication of GBV-B replicon RNA to a level above the detection threshold of the selection system used. The cell morphology, especially in terms of cell size, also showed a certain degree of variability among the selected cell lines and original Huh7 cells, as shown in Fig. 3, particularly in panel C, where cells were photographed with visible light.

Due to the lack of availability of chimeric HCV/GBV-B constructs proven viable in tamarins, it was not possible to gain positive experimental evidence that such chimeric constructs might give coherent results as replicons in cells and as full-

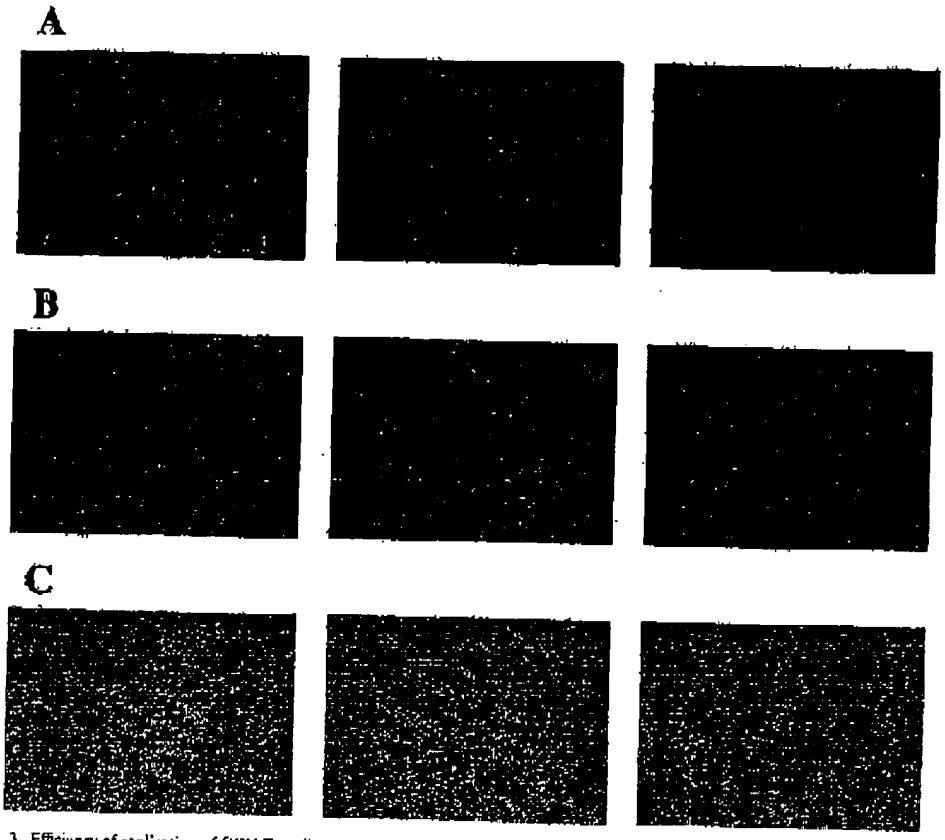


FIG. 3. Efficiency of replication of GBV-B replicons in the normal Huh7 population and in selected Huh7-derived clones. (A) Transfection of bla-RepB RNA in Huh7 (left), cB76.1/Huh7 (middle), and cB76.1/Huh7 cells in the presence of IFN-α (right). (B) Transfection of bla-RepB in Huh7-derived cell lines A20 (left), B57 (middle), and B59 (right) bearing neo-RepB RNA. Cell counting indicated 25, 40, and 10% blue cells for the three cell lines, respectively. In panels A and B, cells were observed with a 10× magnification microscope lens and photographed with UV light at 48 h posttransfection. The blue-stained cells contain detectable β-lactamase levels, and the green-stained cells are background nontransfected cells or cells producing β-lactamase amounts below the detection threshold. (C) Morphology of Huh7-derived cell lines, Huh7 (left), B76 (middle), and B59 (right) cells were stained with CCF2 Aurora substrate and photographed with visible light under 10× magnification.

length genomes in animals. Nonetheless, we could exclude the occurrence of false-negative results in the GBV-B replicon system at least in the case of a chimeric construct that had already been demonstrated to be unable to replicate in vivo as a full-length version (C. Traboni, unpublished data). The chimera was obtained by replacing the GBV-B NS5B gene in the neo-RepB and bla-RepB constructs with the corresponding gene from the H77 strain of HCV, generating neo-RepB/HCVpol and bla-RepB/HCVpol, respectively. We took advantage of the availability of the cB76.1/Huh7 cell line as well as the unselected Huh7 population and of Huh7 cells cured of the HCV replicon (G. Paonessa, unpublished data) for testing both versions of the chimeric construct.

G418 resistance selection, TaqMan RNA measurements, and β-lactamase expression experiments were performed as described for the GBV-B parental constructs. Parental constructs were used as positive controls, and GAA mutants were used as negative controls. In no case did the data obtained with the chimeric RNA differ from those obtained with the nonreplicating GAA mutant, indicating that the particular chimeric RNA tested was unable to replicate, even in enhanced cells, confirming the data obtained in tamarins with full-length RNA.

Effect of antiviral agents on GBV-B replicon. To acquire information about the susceptibility of GBV-B replicon to antiviral agents, in particular to those known to be active against

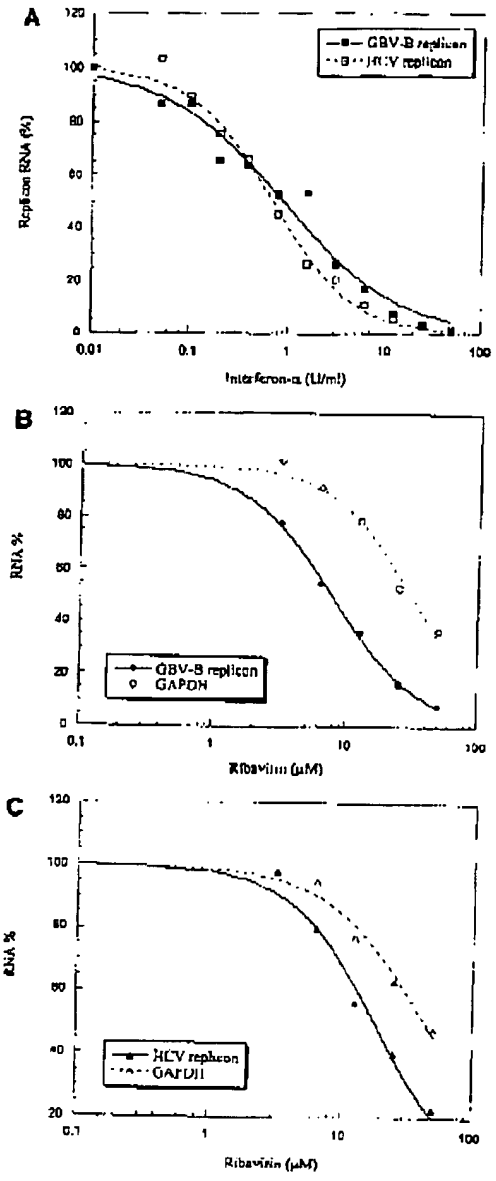


FIG. 4. Effects of antiviral agents on replication of GBV-B and HCV RNA in replicon cell lines. (A) Effect of human IFN-α on both GBV-B (continuous line, solid squares) and HCV (dotted line, open squares) replicon RNAs. The amount of viral RNA was normalized to the amount of GAPDH mRNA. (B) Effect of ribavirin on GBV-B replicon RNA (continuous line, solid circles) compared to the effect produced on GAPDH mRNA (dotted line, open circles). (C) Effect of ribavirin on HCV replicon RNA (continuous line, solid triangles) compared to the effect produced on GAPDH mRNA (dotted line, open triangles). The B76.1/Huh7 and HBI10A cell lines were used to measure GBV-B and HCV replicon RNA levels, respectively. Both replicons and GAPDH RNA values are reported as a percentage of the values obtained in the absence of test compounds. The calculations and plots were generated with the Excel and Kaleidagraph programs.

HCV, we tested the effect of human IFN-α, ribavirin, and other low-molecular-weight chemical compounds on the GBV-B replicon system.

We used the B76.1/Huh7 GBV-B replicon cell line in parallel with the HBI10A HCV replicon cell line (18) as an HCV replication reference system. Cells were grown as described in Materials and Methods in the presence of the test compound or of the corresponding solvent as a control, taking care that confluence was not reached to avoid nonspecific inhibition (21). RNA was extracted, and TaqMan analysis was performed with a primer-probe set specific for the neomycin phosphotransferase gene in order to avoid any methodological difference in the measurement of the HCV and GBV-B RNA molecules. The viral RNA amount was compared to the amount of the endogenous reference mRNA coding for the housekeeping enzyme GAPDH to take into account both experimental variability and, whenever present, cytotoxicity effects.

We first analyzed the effect of human IFN-α (Fig. 4A), the only drug approved for hepatitis C monotherapy and also reported to be active against the HCV replicon (7, 11). We detected comparable inhibition of the GBV-B and HCV replicons, with a 50% inhibitory concentration (IC_{50}) of 0.88 and 0.69 U/ml, respectively, in agreement with the results already available for HCV replicons, and no significant variation in GAPDH RNA levels (data not shown). Analyzing the effect of ribavirin was complicated by cell toxicity, detectable by visualization of cell health status by the MTT colorimetric cell assay (data not shown) and by measurement of GAPDH cellular mRNA. Inhibition of GBV-B replication was also detectable, but in the concentration range in which the toxicity parameters were significantly altered. In Fig. 4B and C, the curves corresponding to replicons and endogenous GAPDH reference RNA were reported separately to show the cytotoxicity effect of ribavirin, detectable by measurement of the mRNA from a housekeeping gene such as GAPDH.

In Fig. 4B, the inhibition curves of ribavirin on the GBV-B replicon and on GAPDH RNA in the B76.1/Huh7 cell line are shown. In Fig. 4C the effect on the HCV replicon and GAPDH in the HBI10A cell line is shown. A separate calculation of the potency of ribavirin on GBV-B and GAPDH RNA in the B76.1/Huh7 cell line gave an IC_{50} of 7.7 and 31, respectively. In the case of HCV and GAPDH RNA in the HBI10A cell line, the values were 18 and 43 μM, respectively. In both cases, apart from the toxicity phenomenon, this might suggest that a moderate specific effect of ribavirin is exerted on viral replication, slightly more pronounced for GBV-B. This was confirmed by calculating the inhibition of viral RNA upon normalization of the direct measure of viral RNA to the amount of the GAPDH internal standard RNA (IC_{50} of 10.6 μM for GBV-B and 43.6 μM for HCV). Mycophenolic acid, which, like riba-

virin, decreases the endocellular pool of dGTP through inhibition of the IMP dehydrogenase, does not seem to be specifically effective on either GBV-B or HCV replication while producing high cytotoxicity, already detectable at concentrations of around 0.2 μM (data not shown). Finally, 4-{[(2*E*)-3-phenylprop-2-enyl]oxy}benzaldehyde thiosemicarbazone, a molecule belonging to a class of compounds active on several viruses, including human immunodeficiency virus and herpes simplex virus (33, 34; S. Altamura, unpublished data), inhibits GBV-B replication, with an IC_{50} of 0.250 μM, showing no appreciable cell toxicity up to 5 μM (data not shown).

DISCUSSION

Autonomously replicating RNA molecules transcribed from cDNA constructs encoding portions of viral genomes can be invaluable substitutes for complete virions when a true infection system is not available, as in the case of HCV. In all cases, replicon molecules are a powerful tool for dissecting the mechanisms underlying replication and other important functions of the virus life cycle performed by nonstructural proteins by means of reverse genetics. A striking example of this is the work based on subgenomic replicons of the pestivirus bovine viral diarrhea virus, which led to an important body of information about the replication and pathogenicity of this virus (3, 9, 10, 22, 32, 35, 36).

In the case of HCV, establishment of a replicon system marked a turning point in the acquisition of important information, as a sound cell-based infection system was and is still not available. Since the first report on an HCV replicon (16), the molecular and cellular features of the system have been better defined, and interesting results that evidence an interplay between viral and host elements continue to emerge (4, 13, 15, 21; G. Paonessa, unpublished data). The system will probably find its most useful application in studying the effects of novel drug candidates on HCV replication. The potential of the HCV replicon in this field is demonstrated by recently reported studies on molecular mechanisms of traditional antiviral agents achieved using this system (7, 11).

We decided to establish a subgenomic replicon-based system for the flavivirus GBV-B mainly to acquire a useful tool for testing HCV/GBV-B chimeric constructs in cells. For the construct design, we followed the route successfully explored for HCV, reproducing the HCV replicon gene arrangement with the same selector gene, neomycin phosphotransferase, fused to the N terminus of the core protein and the EMCV IRES directing the synthesis of GBV-B polyprotein.

We tested the human hepatoma Huh7 cell line as a recipient of GBV-B replicons in parallel with other cell lines, including Hep3B and HepG2 human hepatomas, getting positive results only with the Huh7 cells. The efficiency in generating selectable cell clones bearing GBV-B replicons was, however, very low, in apparent contrast to the results obtained by sequencing the GBV-B replicon from one of the antibiotic-resistant clones, whose sequence was proven identical to that of the input replicon RNA. Since the originally transfected RNA could apparently replicate without adaptive mutations, it was presumed it would replicate in all the transfected cells in the Huh7 population, provided they were equally capable of sustaining replication. The hypothesis we formulated to reconcile the absence of adaptive mutations and low replication efficiency was that, as observed for HCV replicons (G. Paonessa, unpublished data), only a fraction of the transfected Huh7 cells was actually capable of sustaining the replication of GBV-B sequences. We experimentally proved this hypothesis by determining that the low replication efficiency observed with the starting Huh7 population peaked to a 20,000-fold increase when a selected clone was retransfected after removal of resident GBV-B RNA molecules by means of extensive IFN-α treatment.

The possibility that the increased replication was an artifact consequent to IFN treatment can be reasonably discarded according to data obtained with HCV replicon clones showing the same behavior when cured with IFN and with other antiviral molecules (G. Paonessa, unpublished data). Moreover, increased GBV-B replication similar to that obtained with the IFN-cured clone described above was also detected by transfecting cells of the same clone not treated with IFN but simply grown for 20 days without G418 selection (data not shown).

The secondary transfection, performed with bla-RepB RNA, was a powerful tool because it provided a clear and quick direct detection of the number of cells supporting replication. The hypothesis that the enhancement of replication due to host cell factors was a rule rather than an exception was confirmed by the observation that each of several neo-RepB cell lines sustained replication of bla-RepB with a higher efficiency (number of bla-positive cells) than the unselected Huh7 population. The possibility that the enhanced replication observed with this experimental approach is merely due to effects mediated by resident replicon molecules seems to be excluded by the fact that cells of the B76.1/Huh7 cell line do not sustain the replication of newly transfected bla-RepB RNA more efficiently than the corresponding cured cB76.1/Huh7 cells (data not shown).

It also has to be noted that some morphological features of the individual clones, for instance, cell size, appear to be different not only from that of parental Huh7 cells but also among the selected clones (see examples in Fig. 2C). This observation is also consistent with the hypothesis that those clones were actually independently originated and that they did not necessarily enhance GBV-B replication through the same mechanism.

In conclusion, only a subset of cells of the original population have the potential to become resistant clones, provided that they are transfected with RNA producing a sufficient amount of neomycin phosphotransferase in the early post-transfection phases, when the number of RNA molecules is limited. Thus, an interplay between GBV-B replicon RNA "fitness" and unknown features of host cells seems to contribute to establishing the cell lines in which GBV-B RNA replicates. This is confirmed by the observation that a variant of the neo-RepB construct, lacking the region encoding the N terminus of core protein, probably affected in IRES functionality, though enhanced in cB76.1/Huh7, was less efficient in replication than neo-RepB RNA in both Huh7 and enhanced cells. It should also be considered that the phenomenon of cell-based replication enhancement does not necessarily exclude that adaptive mutations of the replicating GBV-B molecules may exist in a minority of cell clones. Combinations of the two effects might account for the variability in the replicon-bearing

cell clones' phenotypes, especially in terms of growth rate and replicon copy number.

In the GBV-B replicon system, however, even in the best experimental conditions, the number of RNA molecules per cell is lower than that of the HCV replicon, which reaches 5,000 (4, 16). From this aspect, the low G418 concentration chosen for the early phases of selection might have been even more important than for the HCV replicon (21), since it allowed the expansion of tiny slow-growing cell clones bearing a small number of molecules that initially produced low levels of neomycin phosphotransferase. As experimentally demonstrated, these clones would have been missed if concentrations higher than 0.250 mg/ml had been used for the initial selection.

An important feature of the GBV-B/Huh7 replication system described is that the sequence of the GBV-B-specific regions of the subgenomic replicons was identical to that of a genomic construct able to infect tamarins (28). This implies that the viral *cis*- and *trans*-acting elements involved in GBV-B replication, which interact with tamarin host cell factors, are also capable of productively interacting with human cell factors without sequence adaptation. This would seem to hint at the possibility that, if humans are actually not susceptible to GBV-B infection, the block may not occur at the replication level, but possibly at earlier stages in the virus infection cycle.

The absence of sequence alterations in the GBV-B replicon functioning in cells indicates that, unlike the first HCV replicon sequence reported used for Huh7 transfection (16), the GBV-B sequence is already "adapted." We noticed that the amino acids conferring adaptation to the HCV replicon (1) were apparently not conserved in the corresponding positions in the GBV-B sequence. An exception is the highly adaptive mutation R2884G, corresponding to residue 465 of the HCV NS5B protein (15), whose cognate GBV-B position is occupied by the "adapted" amino acid glycine. Both the HCV residue (15) and the GBV-B residue (M. Walsh, personal communication) are located in the three-dimensional structure of the NS5B RdRp on the surface of the molecule far from the active site. This suggests that a role might be played by this amino acid in interactions of the RdRp of both viruses with different viral or cellular molecules or with the endoplasmic reticulum membrane system, considering the proximity of this residue to the hydrophobic C-terminal region that, in the HCV RdRp, has been shown to be a transmembrane domain (30).

An important application of a cell-based replication system for GBV-B is to provide a tool to test in cultured cells HCV/GBV-B chimeric subgenomic RNAs whose corresponding genomic version has to be tested in animals. Unfortunately, no in vivo-viable chimera of that kind has been identified so far. Nonetheless, we could verify that RNA from a chimeric construct in which the NS5B gene of GBV-B had been replaced by the HCV counterpart, which had already been injected in tamarins unsuccessfully prior to the availability of the cell-based system described here (C. Traboni, unpublished data), was also unable to replicate in cells. These results demonstrate that, at least in this specific example, false-positive results are not generated in the cell-based replicon system.

Another important application of the system described here is the possibility of analyzing the effects of candidate antiviral agents against HCV on GBV-B replication in cells prior to testing them in animals. We checked the feasibility of this approach by testing known inhibitors of HCV on the GBV-B replication system. IFN-α, the best-characterized antiviral molecule active against HCV, was an equally efficacious agent against GBV-B and HCV replicons, as determined by various experimental approaches (7, 11). The reported efficacy of human interferon against GBV-B replication in tamarin primary hepatocytes infected by the GBV-B viral inoculum was instead rather limited (14). The authors commented that this could be due to potentially reduced recognition between human interferon and tamarin cell receptors and/or effectors, which should not occur in our system, since both protein and host cells are of human origin. This aspect of the GBV-B replicon/Huh7 system is of general interest when running experiments involving comparison of HCV and GBV-B replicons, since cells of the same origin would reduce host-related variability. The specific effect of ribavirin in our system seems, on the contrary, more difficult to ascertain, mainly due to the higher toxicity that we noticed with the Huh7 cell line under our experimental conditions. The nonreplicating tamarin primary hepatocytes in Lanford's model and the human hepatoma cell line used in our system plus the use of different cell toxicity markers probably make it impossible to compare the data.

The GBV-B subgenomic replicon system described in this paper was mainly devised as a tool to examine the viability of HCV/GBV-B chimeras in a convenient cell line and to test the effect of putative antiviral agents. The results clearly demonstrate that this system meets those expectations. We are now working to construct more chimeric replicons bearing both HCV and GBV-B sequences, with the aim of identifying viable constructs through the analysis of replication ability in the enhanced cell lines that we have selected. Work is also in progress to convert the subgenomic GBV-B replicon into a full-length genomic clone in order to increase the potential of the system, providing a tool to perform both true infection and reverse genetics.

ACKNOWLEDGMENTS

We thank Giovanni Migliaccio, Raffaele De Francesco, Elisa Scarselli, Stefania Di Marco, and Riccardo Cortese for helpful discussions, Jay Grobler for kindly providing the plasmid encoding the β-lactamase gene, Fumio Kobune for B95a tamarin cells, Steven Harper for the anti-HCV thiosemicarbazone, and Mauro Cerretani for help with cytotoxicity assays. We are also grateful to Janet Clench for revising the manuscript and to Manuela Emili for artwork.

REFERENCES

1. Bartenschlager, R., and V. Lohmann. 2001. Novel cell culture systems for the hepatitis C virus. Antiviral Res. 52:1–17.
2. Beames, B., D. Chavez, D. Guerra, L. Neevell, K. M. Brinsky, and R. E. Lanford. 2000. Development of a primary tamarin hepatocyte culture system for GB virus B: a surrogate model for hepatitis C virus. J. Virol. 74:11768–11777.
3. Behrens, S. E., C. W. Grassmann, H. J. Thiel, G. Meyers, and N. Tautz. 1998. Characterization of an autonomous subgenomic pestivirus RNA replicon. J. Virol. 72:2364–2372.
4. Blight, K. J., A. A. Kolykhalov, and C. M. Rice. 2000. Efficient initiation of HCV RNA replication in cell culture. Science 290:1972–1974.
5. Bukh, J., C. L. Apgar, and M. Yanagi. 1999. Toward a surrogate model for hepatitis C virus: an infectious molecular clone of the GB virus-B hepatitis agent. Virology 262:470–478.
6. Buitkewicz, N., N. Yao, W. Zhong, J. Wright-Minogue, F. Ingravallo, R. Zhang, J. Durkin, D. N. Standring, B. M. Baroudy, D. V. Sangar, S. M. Lemon, J. Y. Lau, and Z. Hong. 2000. Virus-specific cofactor requirement and chimeric hepatitis C virus/GB virus B nonstructural protein 3. J. Virol. 74:4251–4301.
7. Frese, M., T. Pietschmann, D. Moradpour, O. Haller, and R. Barten-

schlager. 2001. Interferon-alpha inhibits hepatitis C virus subgenomic RNA replication by an MxA-independent pathway. J. Gen. Virol. 82:723–733.
8. Gruez, K., M. Garland, P. Karayiannis, M. J. McGarvey, and H. Clarke. 1999. The 5′ untranslated region of GB virus B shows functional similarity to the internal ribosome entry site of hepatitis C virus. J. Gen. Virol. 80:2237–2241.
9. Grassmann, C. W., O. Isken, and S. E. Behrens. 1999. Assignment of the multifunctional NS5 protein of bovine viral diarrhea virus during RNA replication: an in vivo and in vitro study. J. Virol. 73:9196–9205.
10. Grassmann, C. W., O. Isken, N. Tautz, and S. E. Behrens. 2001. Genetic analysis of the pestivirus nonstructural coding region: defects in the NS5A unit can be complemented in trans. J. Virol. 75:7791–7803.
11. Guo, J. T., V. V. Bichko, and C. Seeger. 2001. Effect of alpha interferon on the hepatitis C virus replicon. J. Virol. 75:8516–8523.
12. Hoofnagle, J. H. 1997. Management of hepatitis C: current and future perspectives. J. Hepatol. 31:264–268.
13. Krieger, N., V. Lohmann, and R. Bartenschlager. 2001. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. J. Virol. 75:4614–4624.
14. Lanford, R. E., D. Chavez, B. Guerra, J. Y. Lau, Z. Hong, K. M. Brasky, and G. Beames. 2001. Ribavirin induces error-prone replication of GB virus B in primary tamarin hepatocytes. J. Virol. 75:8074–8081.
15. Lohmann, V., F. Korner, A. Dobierzewska, and R. Bartenschlager. 2001. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. J. Virol. 75:1437–1449.
16. Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285:110–113.
17. Mercer, D. F., D. E. Schiller, J. F. Elliott, D. N. Douglas, C. Hao, A. Rinfret, W. R. Addison, K. P. Fischer, T. A. Churchill, J. R. Lakey, D. L. Tyrrell, and N. M. Kneteman. 2001. Hepatitis C virus replication in mice with chimeric human livers. Nat. Med. 7:927–933.
18. Mottola, G., G. Cardinali, A. Ceccacci, C. Trozzi, L. Bartholomew, M. R. Torrisi, E. Pedrazzini, S. Bonatti, and G. Migliaccio. 2002. Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. Virology 293:31–43.
19. Muerhoff, A. S., T. P. Leary, J. N. Simons, T. J. Pilot-Matias, G. J. Dawson, J. C. Erker, M. L. Chalmers, G. G. Schlauder, S. M. Desai, and I. K. Mushahwar. 1995. Genomic organization of GB viruses A and B: two new members of the *Flaviviridae* associated with GB agent hepatitis. J. Virol. 69:5621–5630.
20. National Institutes of Health. 1997. Consensus Development Conference Panel Statement: management of hepatitis C. Hepatology 26:2S–10S.
21. Pietschmann, T., V. Lohmann, G. Rutter, K. Kurpanek, and R. Bartenschlager. 2001. Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J. Virol. 75:1252–1264.
22. Qu, L., L. E. McMullan, and C. M. Rice. 2001. Isolation and characterization of noncytopathic pestivirus mutants reveals a role for nonstructural protein NS4B in viral cytopathogenicity. J. Virol. 75:10651–10662.
23. Rijnbrand, R., C. Abell, and S. M. Lemon. 2000. Mutational analysis of the GB virus B internal ribosome entry site. J. Virol. 74:773–783.
24. Rijnbrand, R., P. J. Bredenbeek, P. C. Haasnoot, J. S. Kieft, W. J. Spaan, and S. M. Lemon. 2001. The influence of downstream protein-coding sequence on internal ribosome entry on hepatitis C virus and other flavivirus RNAs. RNA 7:585–597.
25. Rosenberg, S. 2001. Recent advances in the molecular biology of hepatitis C virus. J. Mol. Biol. 313:451–464.
26. Sbardellati, A., E. Scarselli, V. Amati, S. Falcinelli, A. S. Kekulé, and C. Traboni. 2000. Processing of GB virus B nonstructural proteins in cultured cells requires both NS3 protease and NS4A cofactor. J. Gen. Virol. 81:2783–2789.
27. Sbardellati, A., E. Scarselli, L. Tomei, A. S. Kekulé, and C. Traboni. 1999. Identification of a novel sequence at the 5′ end of the GB virus B genome. J. Virol. 73:10340–10348.
28. Sbardellati, A., E. Scarselli, B. Verschoor, A. De Tomassi, D. Lazzaro, and C. Traboni. 2001. Generation of infectious and transmissible virions from a GB virus B full-length consensus clone in tamarins. J. Gen. Virol. 82:2437–2448.
29. Scarselli, E., A. Urbani, A. Sbardellati, L. Tomei, R. De Francesco, and C. Traboni. 1997. GB virus B and heparin C virus NS3 serine proteases share substrate specificity. J. Virol. 71:4985–4989.
30. Schmidt-Mende, J., E. Bieck, T. Hugle, F. Penin, C. M. Rice, H. E. Blum, and D. Moradpour. 2001. Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. J. Biol. Chem. 276:44052–44063.
31. Simons, J. N., T. J. Pilot-Matias, T. P. Leary, G. J. Dawson, S. M. Desai, G. G. Schlauder, A. S. Muerhoff, J. C. Erker, S. L. Buijk, M. L. Chalmers, et al. 1995. Identification of two flavivirus-like genomes in the GB hepatitis agent. Proc. Natl. Acad. Sci. USA 92:3401–3405.
32. Tautz, N., T. Harada, A. Kaiser, C. Rinck, S. Behrens, and H. J. Thiel. 1999. Establishment and characterization of cytopathogenic and noncytopathogenic pestivirus replicons. J. Virol. 73:9422–9432.
33. Taks, Y., N. Barka, M. Abramoff, and D. Ronen. 1994. Relationships between structure and antiretroviral activity of thiosemicarbazone derivatives. Chemotherapy 40:195–200.
34. Teltz, Y., D. Ronen, A. Vansover, T. Stamatsky, and J. L. Riggs. 1994. Inhibition of human immunodeficiency virus by *N*-methylisatin-β-4′,4′-diethylthiosemicarbazone and *N*-allylisatin-β-4′,4′-diallylthiosemicarbazone. Antiviral Res. 24:305–314.
35. Yu, H., C. W. Grassmann, and S. E. Behrens. 1999. Sequence and structural elements at the 3′ terminus of bovine viral diarrhea virus genomic RNA: functional role during RNA replication. J. Virol. 73:3638–3648.
36. Yu, H., O. Isken, C. W. Grassmann, and S. E. Behrens. 2000. A stem-loop motif formed by the immediate 5′ terminus of the bovine viral diarrhea virus genome modulates translation as well as replication of the viral RNA. J. Virol. 74:5825–5835.
37. Zheng, W., P. Ingravallo, J. Wright-Minogue, A. Skelton, A. S. Uss, R. Chase, N. Yao, J. Y. Lau, and Z. Hong. 1999. Nucleoside triphosphatase and RNA helicase activities associated with GB virus B nonstructural protein 3. Virology 263:216–226.
38. Zhong, W., P. Ingravallo, J. Wright-Minogue, A. S. Uss, A. Skelton, R. Ferrari, J. Y. Lau, and Z. Hong. 2000. RNA-dependent RNA polymerase activity encoded by GB virus-B nonstructural protein 5B. J. Viral Hepat. 7:335–342.